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Temporal separation of distinct differentiation pathways by a dual specificity Rap-Phr system in *Bacillus subtilis*

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Summary

In bacterial differentiation, mechanisms have evolved to limit cells to a single developmental pathway. The establishment of genetic competence in *Bacillus subtilis* is controlled by a complex regulatory circuit that is highly interconnected with the developmental pathway for spore formation, and the two pathways appear to be mutually exclusive. Here we show by *in vitro* and *in vivo* analyses that a member of the Rap family of proteins, RapH, is activated directly by the late competence transcription factor ComK, and is capable of inhibiting both competence and sporulation. Importantly, RapH is the first member of the Rap family that demonstrates dual specificity, by dephosphorylating the Spo0F~P response regulator and inhibiting the DNA-binding activity of ComA. The protein thus acts at the stage where competence is well initiated, and prevents initiation of sporulation in competent cells as well as contributing to the escape from the competent state. A deletion of *rapH* induces both differentiation pathways and interferes with their temporal separation. Together, these results indicate that RapH is an integral part of a multifactorial regulatory circuit affecting the cell's decision between distinct developmental pathways.

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Introduction

Bacterial differentiation is accompanied by the synthesis of a dedicated set of proteins. It is therefore energetically unfavourable for single cells to enter more than a single developmental pathway at the same time and from this it follows that mechanisms have evolved to limit cells to a single differentiation process.

The Gram-positive soil bacterium *Bacillus subtilis* is characterized by a complex developmental pathway that, under appropriate conditions of nutrient limitation, may lead to the formation of dormant, environmentally resistant spores (Errington, 1993). However, spore formation may be the final choice among a series of responses that the cell can initiate in an attempt to survive a hostile environment. The alternative responses of *B. subtilis*, aimed at tapping into new resources and eliminating competitors, include synthesis and secretion of degradative enzymes, production of antibiotics, motility and the development of competence for genetic transformation (for reviews see Dubnau and Lovett, 2002; Phillips and Strauch, 2002; Piggot and Hilbert, 2004). Common triggers, such as nutrient limitation and high cell density, have led to the incorporation of shared elements in the regulatory cascades governing these adaptive responses (Hamoen *et al.*, 2003a). For instance, competence and sporulation are negatively affected by the same repressors, CodY and AbrB, during exponential growth, when nutrients are plentiful (Strauch, 1993; Sonenshein, 2005). Additionally, some proteins, like Spo0A, are indispensable for both processes (Hahn *et al.*, 1995a; Fujita and Losick, 2003).

Competence development is a transient process that requires the key transcriptional regulator ComK (Hahn *et al.*, 1994; Van Sinderen *et al.*, 1995). The *comK* gene is subject to complex regulation at the transcriptional as well as the post-translational level (Dubnau and Lovett, 2002; Hamoen *et al.*, 2003a). Sporulation is a last-resort adaptive process, governed by the master regulator Spo0A (Errington, 2003; Fujita and Losick, 2003). In order to exert its effect as a transcriptional regulator, the protein needs to be phosphorylated and the level of phosphorylation is tuned by a series of kinases and phosphatases, including the phosphorelay intermediate Spo0F~P (Burbulys *et al.*, 1991; Perego *et al.*, 1994).

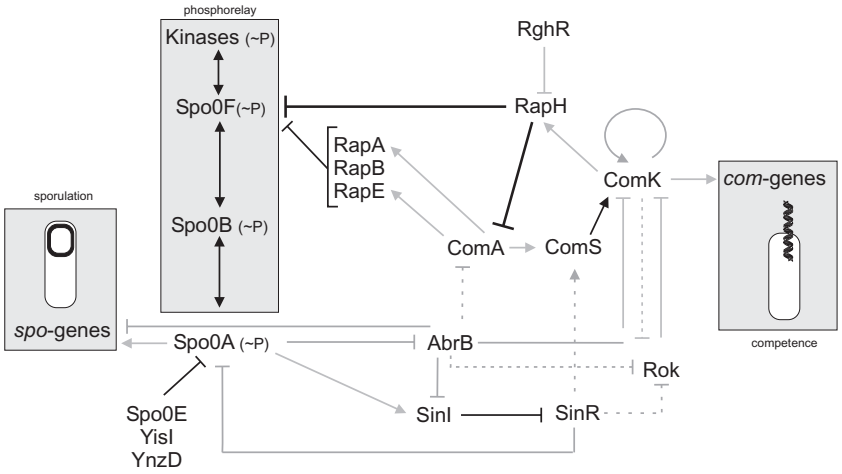

Fig. 1. Regulatory circuit controlling sporulation initiation and competence development. Only selected proteins involved in the interconnected pathways regulating competence development and sporulation initiation are shown. Arrows indicate positive control while perpendiculars indicate negative control. Black lines represent regulation by protein–protein interaction and grey lines represent regulation at the level of gene transcription. Dotted lines indicate regulatory effects defined by genetic analyses but whose mechanism is still unknown. Bold lines indicate the results from this study.

Early events in the competence regulatory cascade involve the activation of the essential competence gene *comS* by the transcription factor ComA (Nakano *et al*., 1991; Hamoen *et al*., 1995). At the same time, ComA activates the expression of two phosphatases, RapA and RapE, which inhibit sporulation (Mueller *et al*., 1992; Jiang *et al*., 2000). Therefore, it is believed that cells that have entered competence development do not enter sporulation. Besides regulation at the transcriptional level, the activity of Rap proteins can be antagonized by pentapeptides that are encoded by *phr* genes (Perego and Brannigan, 2001). The *rap* and *phr* genes are genetically linked, although a *phr* gene could not be identified for all *rap* genes (Perego and Hoch, 2002).

High levels of phosphorylated Spo0A lead to the induction of sporulation genes, but also activation of the *sinI* gene and repression of *abrB* (Perego *et al*., 1988; Shafikhani *et al*., 2002). *sinI* encodes the antagonist for SinR, which is both a negative regulator for sporulation and a positive requirement for competence development (Bai *et al*., 1993; Mandic-Mulec *et al*., 1995). AbrB is a negative regulator of competence (Hamoen *et al*., 2003b), but for reasons poorly understood is also required for this process (Hahn *et al*., 1994; Hahn *et al*., 1995a). Therefore, once a cell has reached a high level of Spo0A~P, competence is prevented and sporulation is stimulated. A schematic representation of this complex regulatory circuit is depicted in Fig. 1.

Upon prolonged nutrient limitation, it can be envisaged that competent cells progress into sporulating cells. Indeed, the formation of spores from previously competent cells has been shown (De Lencastre and Piggot, 1979; Veening *et al*., 2006). As competent cells are metabolically inert (Nester and Stocker, 1963), this requires a return to vegetative growth. If the two pathways are not mutually exclusive from the first stage on, it suggests the existence of a mechanism that ensures a delay of sporulation in competent cells. Previously identified mechanisms act either before competence development is in full swing (RapA and RapE) or at the stage where sporulation gene expression already occurs (SinR and SinI).

In this report, we identify a novel factor involved in the temporal separation of competence and sporulation. RapH, a member of the Rap family of proteins, is specifically activated by the late competence transcription factor ComK. RapH is the first member of this family that has a dual specificity by acting on two distinct response regulators. It promotes the dephosphorylation of Spo0F~P and inhibits the DNA-binding activity of ComA. RapH thus exerts a negative feedback control on early competence events that may contribute to the escape from the competent state, as originally postulated by Hahn *et al*. (1994) and, importantly, prevents sporulation as long as competence prevails.

Results

ComK activates rapH *by reversing RghR repression*

Previous studies suggested a direct role for the late competence transcription factor ComK in the regulation of the *rapH* gene (Berka *et al*., 2002; Hamoen *et al*., 2002; Ogura *et al*., 2002). *In vivo* studies revealed a fourfold reduction of *rapH* transcription in a *comK* mutant compared with a wild-type strain (Fig. 2A and Hayashi *et al*., 2006). *In vitro* binding experiments demonstrated that the affinity of ComK for the *rapH* promoter (K_D ~150 nM) is comparable to the affinity observed for the well-characterized ComK-target promoter *comG* (K_D ~100 nM) (Susanna *et al*., 2004), confirming that the induction is most likely the result of direct binding of ComK to the *rapH* promoter region (Fig. 2B).

The product of the *yvaN* gene (now renamed *rghR*) was recently shown to act as a repressor of *rapH* (Hayashi

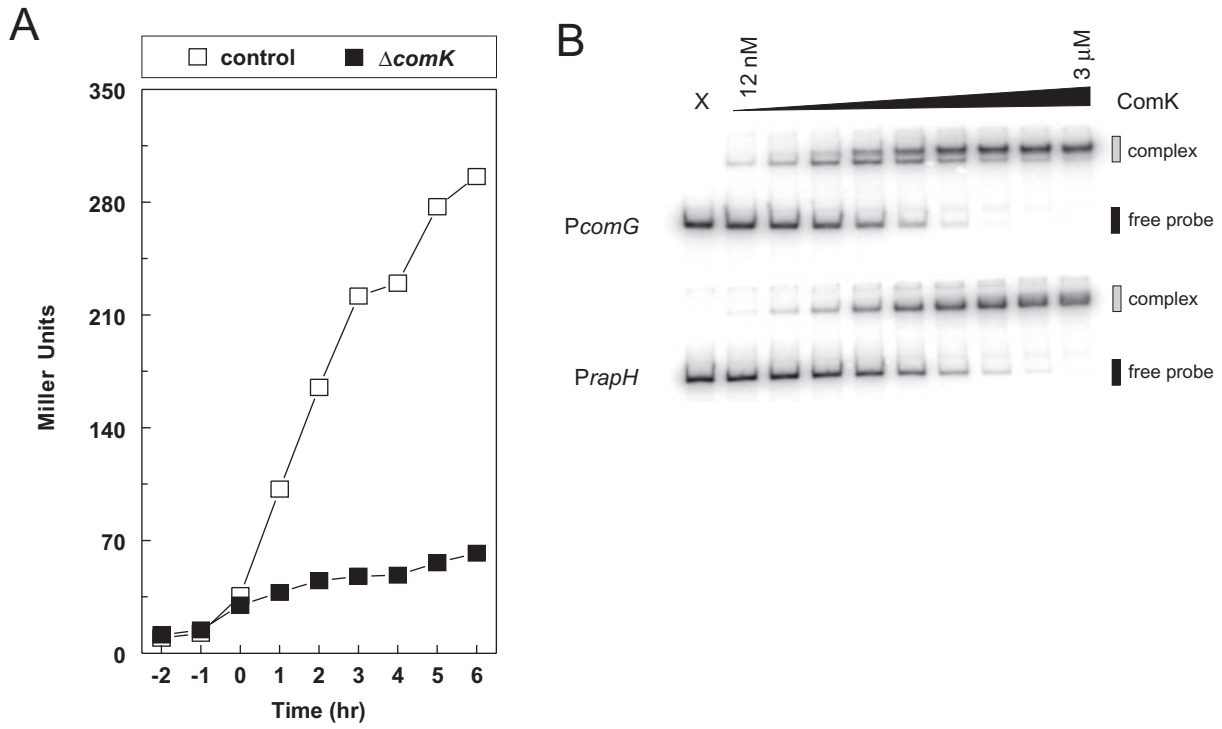

Fig. 2. ComK regulation of *rapH* transcription.
A. Time-courses of β-galactosidase activity of the *rapH–lacZ* transcriptional fusion in the parental strain (-□-) or in the *comK* deletion strain (-■-).
B. Electrophoretic mobility shift assays were carried out in the presence of purified ComK using [γ-^{32}P]-ATP-labelled fragments of the promoter regions of *rapH* (P*rapH*) and *comG* (P*comG*). Fragments were incubated with increasing amounts of purified ComK protein. Grey bars indicate shifted complexes. Black bars indicate unshifted probe. X marks the lane to which no protein was added.

et al., 2006). To determine whether any epistatic relationship existed between ComK and RghR with respect to *rapH* regulation, we assessed the pattern of *rapH* transcription in single cells by means of a translational fusion of *rapH* to an improved version of the cyan fluorescent protein (*icfp*) (Veening *et al*., 2004). The expression of *rapH–icfp* was compared with that of the ComK-controlled *comG* promoter in the same cells by analysing a *comG–yfp* (yellow fluorescent protein) fusion (Fig. 3) in strains singly or doubly mutated for *comK* and *rghR*. In a wild-type strain *rapH* and *comG* are coexpressed, whereas expression of both *rapH* and *comG* is completely abrogated in a δ*comK* strain. In an *rghR* mutant, 100% of the cells express *rapH*, independently of *comK*. Sporadically (< 1%) these cells also demonstrate fluorescence from the *comG–yfp* fusion, dependent on the presence of *comK*. When ComK was ectopically expressed from an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible promoter, both *comG* and *rapH* fusions were expressed in all cells, independent of the presence of *rghR* (data not shown). Importantly, expression of *rapH* was not detected in a rich medium that does not sustain competence development (TY) unless the *rghR* gene was deleted or *comK* was artificially induced (Fig. 3 and data not shown).

These results suggested that *in vivo* ComK acts as an antirepressor, by reversing the repression exerted by RghR at the *rapH* promoter. It was proposed that RghR recognizes a 5′-ATTGAC-3′ motif, which occurs as an inverted repeat and a single motif in the *rapH* promoter (Hayashi *et al*., 2006). The latter motif overlaps with the ComK box, raising the possibility that ComK eliminates binding of the repressor protein. Electrophoretic mobility shifts demonstrated that the addition of each one of the proteins results in a reduced mobility of the P*rapH* probe, as expected. Interestingly, the addition of RghR protein to mixtures that already contain ComK protein leads to a supershifted complex (Fig. S1). This indicates that both proteins can bind simultaneously to the P*rapH* fragment that was used as a probe, and suggests that they, at least partially, recognize different surfaces of the DNA helix.

Genome-wide analysis of RapH overproduction reveals an effect on competence and sporulation

Recently, the effects of an *rghR* mutation on ComA-dependent gene expression were attributed to RapG and RapH overproduction (Hayashi *et al*., 2006). However, this analysis was performed under conditions where the

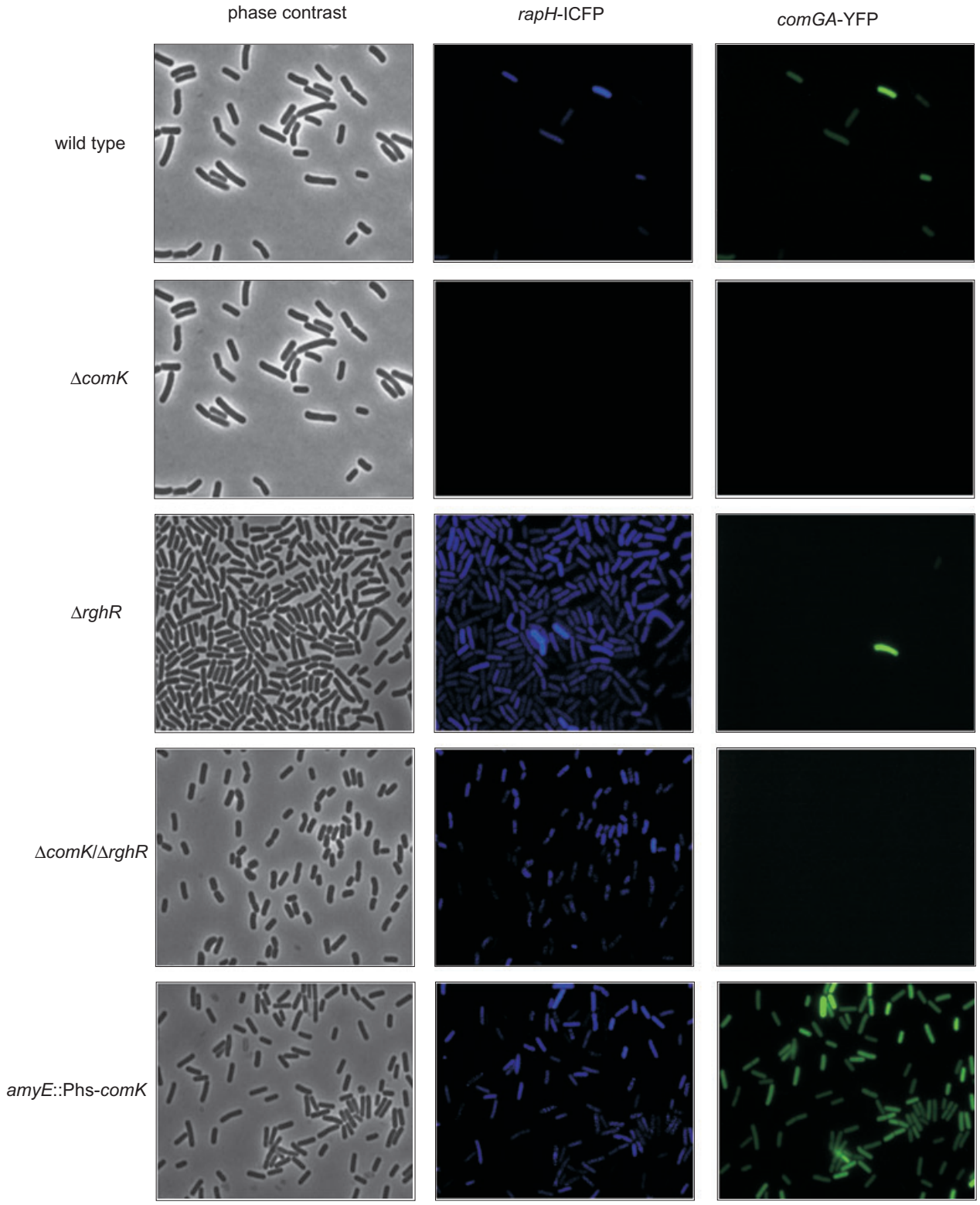

Fig. 3. Fluorescence microscopy of a *rapH–icfp comGA–yfp* strain in *comK* and/or *rghR* mutants. Fluorescence of both reporters was visualized as described in *Experimental procedures*. Δ*comK* indicates the samples from the isogenic *comK*::sp strain, Δ*rghR* indicates the isogenic *yvaN*::tet strain and *amyE*::Phs-*comK* indicates an isogenic strain in which *comK* is expressed from an IPTG-inducible promoter (Maamar and Dubnau, 2005). All strains were grown in minimal medium until 2 h after entry into stationary growth phase, except the Phs-*comK* strain, which was grown in sporulation medium. Induction of *comK* expression from the *amyE*::Phs-*comK* locus was accomplished by the addition of 200 μM of IPTG, and cells were visualized after an additional 2 h of growth.

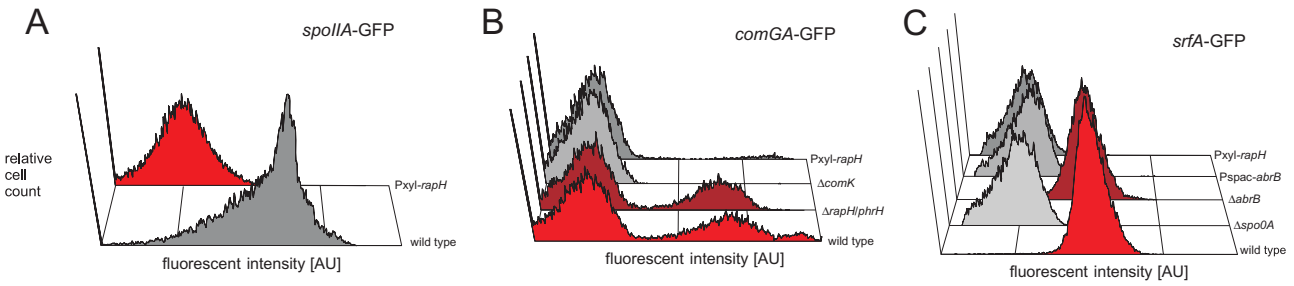

Fig. 4. Flow cytometric analysis of competence and sporulation reporter strains. Analyses were performed as described in *Experimental procedures*.
A. P*spoIIA–gfp* reporter strains were grown in SM medium supplemented with 1% xylose until 3 h after entry into stationary growth phase.
B. P*comG–gfp* reporter strains were grown in MMF supplemented with 1% xylose until 2 h after entry into stationary growth phase.
C. P*srfA–gfp* reporter strains were grown in MMF supplemented with 1% xylose until 2 h after entry into stationary growth phase.
Fluorescence is indicated in arbitrary units (AU) on a logarithmic scale.

rapH gene is normally not significantly expressed and Spo0A-dependent regulation is limited. Furthermore, some of the observed effects may be caused directly by RghR rather than via RapG and/or RapH. Therefore, we mapped the genome-wide transcriptional effects of the RapH/PhrH overproduction 3 h after entry into stationary growth phase in both minimal and sporulation medium. For these experiments, RapH/PhrH overexpression was obtained from a xylose inducible promoter (see *Experimental procedures*). Raw and normalized data of these experiments, including a list of genes significantly affected by RapH-PhrH overproduction, are available from the secure website http://molgen.biol.rug.nl/publications/rapH_data/.

Overexpression of RapH/PhrH significantly affected as much as 9% and 41% of all *B. subtilis* open reading frames (ORFs) in minimal and sporulation medium respectively. A more stringent cut-off (threefold difference, Bayes-$P < 0.0001$) still identified 3% and 23% of the ORFs to be differentially expressed. We correlated the observed regulatory effects to known regulons, derived from the DBTBS database (Makita *et al*., 2004) using the in-house developed FIVA software (Blom *et al*., 2007). This analysis showed that in minimal medium, RapH-PhrH primarily affected the expression of the ComA and ComK regulons (Fig. S2). In sporulation medium, the overproduction of RapH-PhrH led to a very clear negative effect on sporulation gene expression, exemplified by the identification of sporulation specific regulons (e.g. SigE, SigF, SigG, SigK, SpoIIID and SpoVT). In addition, a transcriptional response was observed for many of previously identified target genes for Spo0A (Molle *et al*., 2003; Fujita *et al*., 2005), but due to the absence of these genes as entries in the DBTBS these are not represented in Fig. S2.

Together, these results indicate that overexpression of RapH/PhrH is sufficient to explain the majority of the transcriptional responses observed in the *rghR* mutant (Hayashi *et al*., 2006). Moreover, the data show an effect not only on competence, as previously suggested (Hayashi *et al*., 2006), but also on sporulation gene expression.

Flow cytometric analysis of competence and sporulation reporters

As the negative effects exerted by Rap proteins on these processes are generally more pronounced upon protein overproduction, we conducted flow cytometric analyses of green fluorescent protein (GFP) reporter fusion strains harbouring the ectopic xylose inducible *rapH/phrH* locus (XH) previously used for the DNA array analysis.

The single-cell analyses confirmed that upon overproduction of RapH/PhrH the expression of early (*srfA–gfp*) and late (*comGA–gfp*) competence genes as well as a stage II sporulation gene (*spoIIA–gfp*) was drastically reduced compared with the parental strain (Fig. 4). Importantly, in the absence of xylose there was no detectable difference between the strains (data not shown). The observed inhibition of *spoIIA* promoter activity was consistent with an observed sporulation-deficient phenotype on Schaeffer's sporulation agar plates (see below). The absence of fluorescence in the *comGA* reporter strain resembled a *comK* mutant strain, while the *srfA* reporter strain showed a single-cell profile comparable to a strain deleted for the *spo0A* gene or overproducing the AbrB transition state regulator (Fig. 4). Notably, there was no detectable difference between the flow cytometric profiles of a *comGA–gfp* or *comK–gfp* strain and their *rapH* mutant derivatives during competence development (Fig. 4B and data not shown).

In summary, these results validate the effects observed in the DNA array analysis, but do not address the effects of the *phrH* that is co-transcribed with the *rapH* gene (Hayashi *et al*., 2006).

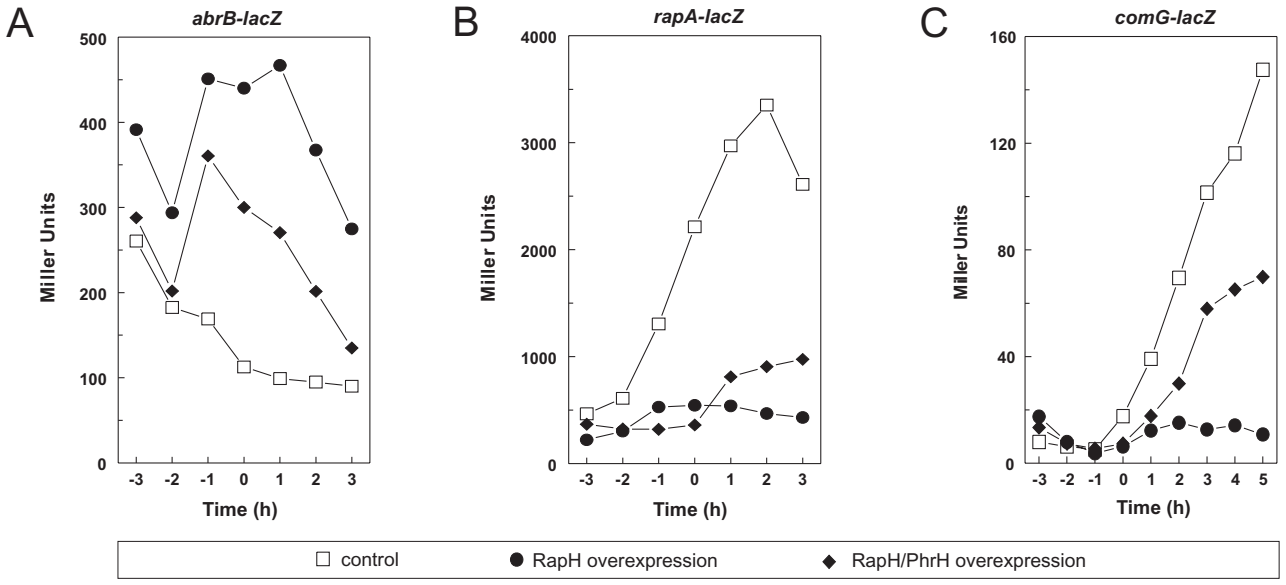

Fig. 5. Transcriptional analysis of competence and sporulation genes. In RapH-PhrH-overexpressing strains, time-courses of β-galactosidase activity were taken at hourly intervals before and after the transition from vegetative growth to stationary phase (T0).
A. *abrB–lacZ* fusion-carrying strains grown in SM medium.
B. *rapA–lacZ* fusion-carrying strains grown in SM medium.
C. *comG–lacZ* fusion-carrying strains grown in Spizizen minimal medium.
Symbols: -□-: strains carrying plasmid control pBS19; -●-: strains carrying plasmid pBS19-RapH2 expressing RapH only; -◆-: strains carrying pBS19-RapH3 expressing RapH and PhrH.

RapH is antagonized by PhrH and inhibits the formation of Spo0A~P

Fragments carrying the *rapH* or the *rapH* and *phrH* genes preceded by the native promoter (209 bp of upstream sequence) were inserted into the multicopy pBS19 plasmid (Bron, 1990), resulting in pBS19-RapH 2 and pBS19-RapH3 respectively. On Schaeffer's sporulation agar plates, the production of RapH from this plasmid led to a clear sporulation-deficient phenotype, whereas this appeared less drastic when PhrH was coexpressed. This observation confirms that PhrH acts as an inhibitor of the RapH protein.

Quantitative transcriptional analyses to investigate this effect in more detail were carried out on strains carrying a phosphorelay reporter gene (*abrB–lacZ*), a ComA-dependent reporter gene (*rapA–lacZ*) and a ComK-dependent reporter gene (*comG–lacZ*) (Fig. 5). The *abrB* gene provides a good qualitative indication of the phosphorylation state of the phosphorelay due to its negative regulation by Spo0A (Perego *et al*., 1988; Jiang *et al*., 2000). As shown in Fig. 5A, expression of RapH resulted in consistently higher levels of *abrB* transcription compared with the control strain. The coexpression of PhrH slightly reduced the overall level of *abrB* expression. A flow cytometric analysis of an *abrB–gfp* reporter strain confirmed that the normal progressive stationary-phase decline in *abrB* transcription was prevented when *rapH-phrH* was induced (Fig. S3).

The *rapA* and *comG* genes are faithful reporters for ComA and ComK activity respectively (Mueller *et al*., 1992; Van Sinderen *et al*., 1995; Jiang *et al*., 2000; Smits *et al*., 2005a). Results obtained from the strain containing a *rapA–lacZ* or a *comG–lacZ* reporter construct demonstrated that the transcription from both promoters was strongly inhibited by RapH overexpression (Fig. 5B and C). Strikingly, coexpression of PhrH with RapH did not significantly effect *rapA* expression, whereas it restored *comG* expression to almost 50%. These results demonstrate that PhrH can counteract the activity of RapH, although full inhibition of RapH activity was not achieved under all conditions. Moreover, RapH evidently inhibits sporulation at its first stage (the repression of *abrB* transcription), as well as early and late competence development.

We considered two possible scenarios that explain the results from the reporter assays: (i) RapH acts on the phosphorelay at the level of the Spo0F intermediate, as previously reported for RapA, B and E (Perego *et al*., 1994; Jiang *et al*., 2000). In this case, the abrogation of competence gene expression would be the result of insufficient inhibition of AbrB production, as this is a requirement for competence development (Hahn *et al*., 1995a). (ii) RapH directly inhibits both the competence pathway

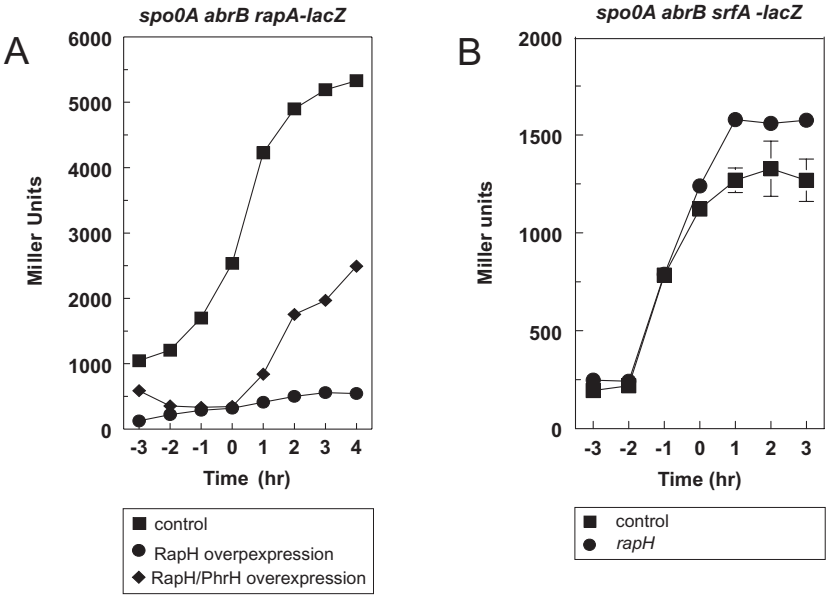

Fig. 6. RapH affects ComA-dependent gene expression independently of sporulation.
A. Time-courses of β-galactosidase activity of a *rapA–lacZ* transcriptional fusion in a *spo0AabrB* double mutant strain carrying the plasmid control pHT315S (-■-), the RapH-expressing plasmid pHT315S-RapH2 (-●-) or the RapH-PhrH-expressing plasmid pHT315S-RapH3 (-◆-).
B. Time-courses of β-galactosidase activity of a *srfA–lacZ* transcriptional fusion in a *spo0AabrB* double mutant strain (-■-) also carrying the deletion of *rapH* (-●-). Cells were grown in SM medium and time points were collected at hourly intervals before and after the transition between vegetative growth and stationary phase (T0). The data presented reflect four independent experiments carried out in duplicate. Error bars for the mutant are too small to be visible.

and the sporulation pathway (via both ComA and Spo0F~P). The possibility that RapH acts on ComA alone, as might be expected based on previous reports (Hayashi *et al*., 2006), was dismissed *a priori*, as overexpression of the RapC and RapF proteins, which specifically inhibit DNA binding of ComA, or the deletion of *comA* does not inhibit sporulation initiation (M. Perego, unpubl. obs.).

RapH negatively affects ComA activation independently of the phosphorelay

As high levels of *abrB* transcription due to RapH overexpression could account for the inhibition of ComA-dependent gene activation (as induction of a Pspac-*abrB* locus abolishes the expression of *srfA–gfp*; see Fig. 4C), we determined whether the RapH effect on competence gene expression was indirect or direct.

Fragments carrying the *rapH* or *rapH* and *phrH* ORFs were cloned into the multicopy shuttle vector pHT315S (Worner *et al*., 2006), yielding pHT315S-RapH2 and pHT315S-RapH3 respectively. These plasmids were transformed into a *spo0A* mutant also lacking the *abrB* gene (*spo0A*12 δ*abrBCm*). In such a strain, competence development is uncoupled from the regulatory effect brought about by the phosphorelay (Hahn *et al*., 1995a and Fig. S5), and thus allows the investigation of an effect directly on ComA (and ComK). As shown in Fig. 6A, overexpression of RapH severely inhibited the induction of *rapA* expression (~10-fold), while a two-fold inhibition was observed in the strain coexpressing RapH and PhrH.

We also analysed the effects of a deletion of *rapH* on ComA-dependent gene expression. When a *srfA–lacZ* reporter fusion was analysed in an otherwise wild-type strain, its transcription was not significantly affected by the *rapH* mutation. This is in agreement with the observation that the transformation efficiency of a *rapH* mutant strain did not differ from the efficiency of the wild-type strain (data not shown) and previous reports (Hayashi *et al*., 2006). We reasoned that the overlapping and contrasting effects due to other Rap proteins on the phosphorelay (RapA, B and E) could mask the effect resulting from the lack of RapH, if this were moderate. Therefore, we assessed the transcriptional profile of a *srfA–lacZ* reporter fusion in a *spo0A abrB* double mutant strain. Figure 6B shows that RapH negatively affects ComA-activated gene expression independently of the phosphorelay, as the *spo0A abrB rapH* triple mutant shows a slight but consistent higher level of *srfA–lacZ* expression than the control strain.

RapH thus not only seems to affect the phosphorelay, but independently also acts on ComA. As RapH is transcriptionally controlled by ComK, this establishes a negative feedback loop in the competence regulatory network as originally postulated to exist by Hahn *et al*. (1994). Importantly, this feedback mechanism can only exert its influence in cells that have already entered competence, and a *rapH* mutation therefore does not result in a significantly altered fraction of competent cells compared with wild-type cells grown in minimal medium (Fig. 4B).

RapH is a dual-function protein targeting both Spo0F~P and ComA

In order to prove a direct role of RapH in modulating the phosphorylation level of the sporulation phosphorelay, we purified the protein from an *Escherichia coli* overexpression strain and tested it *in vitro* against the Spo0F protein that had been phosphorylated by the *B. subtilis* KinA his-

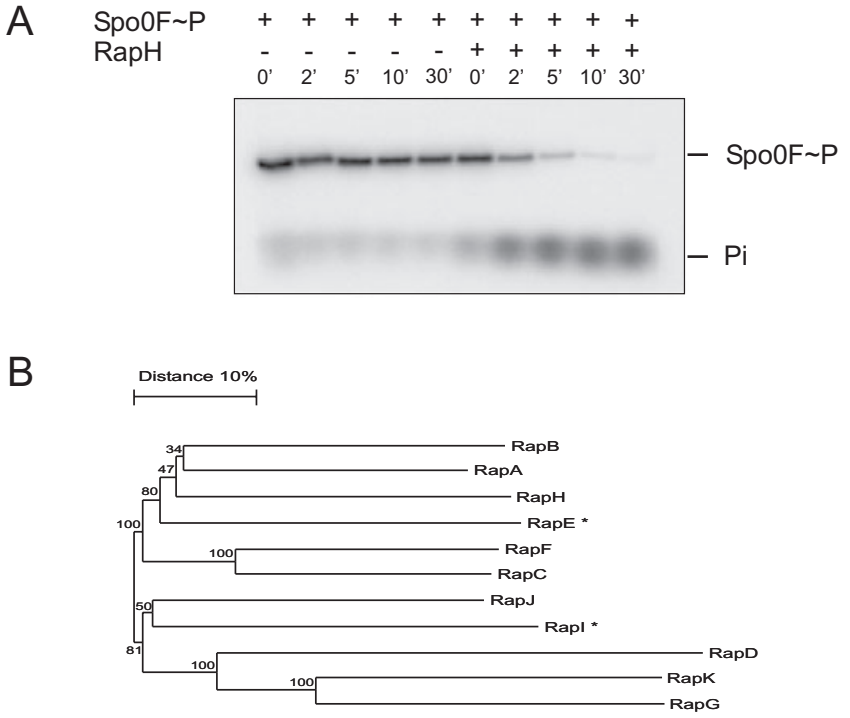

Fig. 7. RapH promotes the dephosphorylation of the Spo0F~P response regulator and clusters with phosphorelay phosphatases.
A. Purified, phosphorylated Spo0F (2 μM) was incubated in the presence or absence of RapH (4 μM) for the time indicated. The positions of the Spo0F protein and the inorganic phosphate released by the reaction are indicated. The samples were run on a 15% SDS polyacrylamide gel.
B. Evolutionary tree of Rap proteins. Asterisks indicate Rap proteins encoded on mobile elements or prophage regions of the *B. subtilis* chromosome (Jiang *et al*., 2000; Auchtung *et al*., 2005).

tidine kinase. Figure 7A shows that RapH can promote the dephosphorylation of Spo0F~P. This result concurs with the bioinformatics observation that RapH shares the highest level of similarity with RapA (47% identity) and clusters in an evolutionary tree with RapA, RapB and RapE (Fig. 7B), which have been shown to target Spo0F~P (Perego *et al*., 1994).

To ascertain a direct role for RapH in inhibiting ComA-dependent transcription, we carried out an *in vitro* binding assay under native conditions as previously done to demonstrate the interaction of ComA with its negative regulators RapC and RapF (Core and Perego, 2003; Bongiorni *et al*., 2005). As shown in Fig. 8A, purified RapH interacted with the full-length ComA protein as indicated by the protein smear in lane 5; RapH also interacted with the C-terminal DNA-binding domain of ComA as suggested by the smeared and retarded band in lane 7, but not with its N-terminal response regulator domain. Consistent with this, RapH inhibited the binding of ComA to a DNA fragment containing the *rapC* promoter in a gel mobility shift assay (Fig. 8B). The ability of RapH to bind ComA is unique among the Rap proteins known to date to act as phosphatases of Spo0F~P; RapA, for example, does not bind to ComA in the native gel binding assay (Fig. S4A), nor does it inhibit binding of ComA to DNA carrying the *rapC* promoter (Fig. S4B). Notably, we additionally established that RapH does not promote dephosphorylation of ComA~P (data not shown).

As it was suggested that RapH might target DegU (Hayashi *et al*., 2006), we also carried out a native gel binding assay using purified RapH and DegU proteins, but no interaction was detected (Fig. S4C).

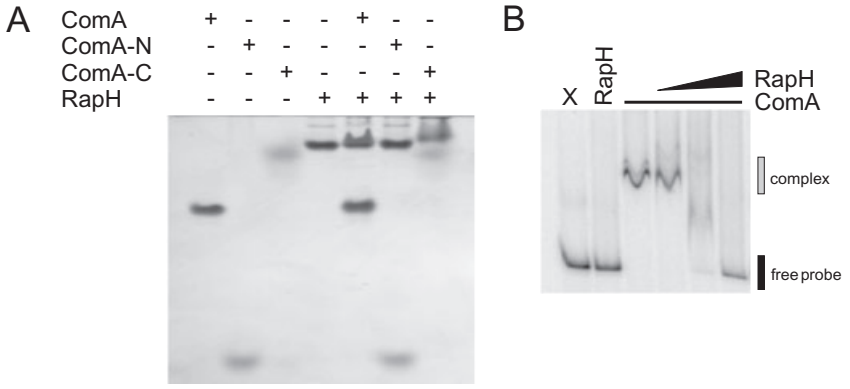

Fig. 8. RapH interacts with ComA and inhibits its DNA-binding activity.
A. Native gel binding assay carried out with purified RapH and ComA; each protein was at 20 μM final concentration. Native gel analysis was carried out on 10% native Tris-Tricine gels as described in Bongiorni *et al*. (2005).
B. Gel mobility shift assay of ComA binding to the *rapC* promoter in the presence or absence of RapH. X: labelled probe only; RapH (lane 2: 5 μM, triangle: 5, 10 and 20 μM); ComA (5 μM).

RapH regulates the temporal separation of competence and sporulation

Under laboratory conditions, competence and sporulation do generally not occur simultaneously (Veening *et al*., 2006); however, in nature, conditions may occur that trigger both processes. Given that competence is a transient differentiation process (Dubnau and Lovett, 2002; Suel *et al*., 2006), whereas sporulation is irreversible from stage II on (Errington, 1993; Dworkin and Losick, 2005), competent cells may have developed strategies to delay sporulation while favouring DNA uptake. The ComA-dependent activation of phosphatases of the Spo0F component of the phosphorelay (RapA and RapE) was originally proposed as one of these strategies (Perego *et al*., 1994; Jiang *et al*., 2000). Here, we showed that ComK-activated RapH negatively regulates sporulation initiation and ComA-dependent competence gene expression. This suggested that RapH might affect the temporal separation of these two processes by a twofold mechanism. First, the protein prevents the expression of sporulation genes in competent cells. Second, RapH imposes negative feedback on competence by the ComK-activated inhibition of ComA, and might contribute to the escape from the competent state. Based on this hypothesis, we predicted that the expression of late-competence genes would be distinct from the sporulation gene expression in a wild-type strain but might overlap in a *rapH* mutant. Indeed, under conditions that sustained both competence and sporulation (see *Experimental procedures*) a strong negative correlation was observed between *comG* (*comG–cfp*) and *spoIIA* (*spoIIA-iyfp*) in wild-type cells (Fig. 9). In contrast, there was a significantly higher amount of cells that simultaneously expressed both reporters in a *rapH* mutant background whereas the frequency of cells expressing either one of the reporters was comparable between wild type and *rapH* mutant (*comG–cfp*: 10.7 ± 2.5 and 8.5 ± 1.2, *spoIIA–iyfp*: 6.6 ± 1.4 and 5.1 ± 1.0, wild type and *rapH* mutant respectively). The data were quantitatively analysed by determining the fraction of fluorescent cells in one channel that also demonstrated fluorescence in the other channel (Fig. 9B). The results indicated that approximately 30% of *comG*- and *spoIIA*-expressing cells also expressed the other reporter in the mutant while only 5% of coexpression was observed in the wild type.

Overall, these data indicate that in a *rapH* mutant sporulation initiation and competence development are no longer strictly separated and certain cells initiate both pathways at the same time.

Discussion

The ComK transcription factor has been defined as the key transcriptional regulator of genetic competence in *B. subtilis* (Van Sinderen *et al*., 1995; Hamoen *et al*., 1998). The identification of genes that are differentially expressed between wild-type and *comK* mutant cells but have no documented function in competence has led to the introduction of the general term K-state to encompass all the physiological effects exerted by ComK (Berka *et al*., 2002; Hamoen *et al*., 2002). Here we identify a novel function for ComK-activated genes, i.e. to ensure appropriate timing between competence and other developmental pathways. Based on the presence of several putative regulators among the core ComK regulon (Hamoen *et al*., 2002) we postulate that other ComK-regulated genes have a role in this interplay.

In addition to activating the expression of the late competence genes and other genes that together result in the establishment of the K-state, ComK also stimulates its own transcription (Van Sinderen and Venema, 1994). Recently, it was found that ComK autostimulation is both necessary and sufficient to explain the bistable expression pattern of the late competence genes (Maamar and Dubnau, 2005; Smits *et al*., 2005a; Smits *et al*., 2006). The transiency of the competent state was first described in 1963, when it was found that the return from metabolically inert competent cells to vegetative growth occurs 3–5 h after the onset of competence (Nester and Stocker, 1963). Transiency can be achieved by the induction of a negative regulator of competence development. Hahn and co-workers observed repression of early competence gene (*srfA-comS*) expression (Hahn *et al*., 1996), and showed that interference with the proteolytic pathway, which includes ComS, interferes with the escape from the competent state (Hahn *et al*., 1995b). Subsequently, it was postulated that ComK-dependent negative feedback on *srfA* expression is involved in the escape from the competent state (Suel *et al*., 2006). Here, we provided the first mechanistic evidence that the negative feedback loop exists by showing that RapH is able to interact with ComA and thus inhibits *srfA* expression (Figs 4C, 6B and 8A and B). Under the conditions tested, the *in vivo* effect of RapH on ComA-dependent gene expression was found to be minor, but it could clearly be demonstrated in a *spo0A abrB* mutant strain. It remains to be established whether native levels of RapH result in a downregulation of *srfA in vivo*, or merely act as a limiter for ComA-dependent gene expression.

RapH is a member of the Rap family of proteins (Perego and Brannigan, 2001). It is the first member of this family for which dual target specificity is documented, as it promotes the dephosphorylation of the phosphorelay intermediate Spo0F~P, next to binding to ComA. The dual specificity of the protein is also reflected in the evolutionary tree based on the amino acid sequences of all members of the Rap family (Fig. 7B). The protein shares the highest homology with the phosphorelay phos-

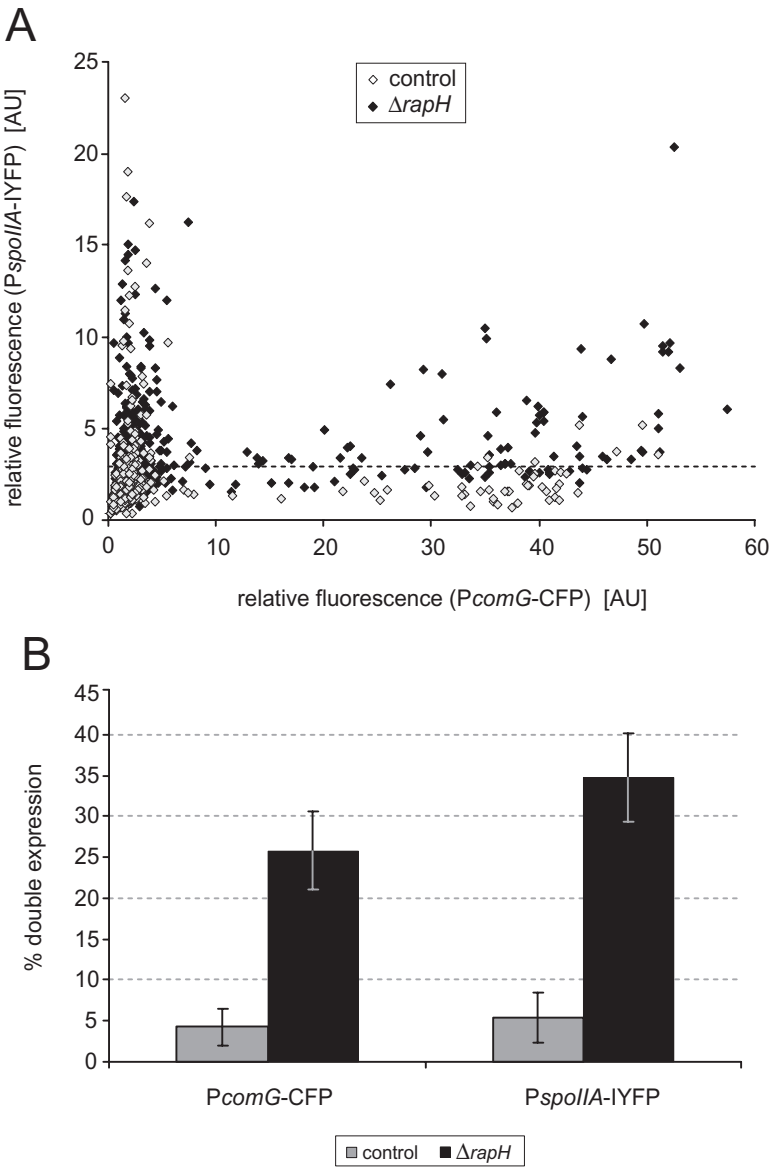

Fig. 9. RapH contributes to the temporal separation of competence and sporulation.
A. Relative fluorescent intensities in the CFP and YFP channels for wild-type and *rapH* mutant cells. Each dot represents a single cell. Ninety-five per cent of the cells that highly express P*comG*–CFP in a wild-type strain demonstrate P*spoIIA*–lYFP fluorescence below the dotted line.
B. The fraction of cells that express both reporters compared with the total number of cells that express either P*comG*–CFP or P*spoIIA*–lYFP. Error bars indicate the standard error of the measurements.

phatases RapA, RapB and RapE, and then with the ComA-interacting RapC and RapF proteins. The other Rap proteins [RapG (Ogura *et al*., 2003), RapI (Auchtung *et al*., 2005), RapD, RapJ and RapK] are more distantly related. It is unknown how the target recognition is achieved, but it is likely that the six TPR domains that structurally characterize the Rap family of proteins are involved (Perego and Brannigan, 2001).

These domains are also believed to be responsible for the interaction between the pentapeptide inhibitors of Rap proteins, which are encoded by the *phr* genes and have been identified for most Rap proteins (Perego and Brannigan, 2001). In some aspects of this study we used overexpression constructs containing the complete *rapH* transcriptional unit, which comprises a *rapH* and *phrH* gene, as independently found by us and others (Hayashi *et al*., 2006). Whereas several *phr* genes can be transcribed from a secondary promoter, *phrH* seems to form a single transcript with *rapH* only (McQuade *et al*., 2001; Hayashi *et al*., 2006). Although we could demonstrate that PhrH can counteract the inhibition imposed by RapH *in vivo* (Fig. 5), the effects were moderate and subject to variations in media and growth conditions. This may be the result of differential mRNA stability or the efficiency of import, export and processing of the PhrH protein into the inhibitory peptide. Thus, the effects observed in the array and flow cytometric analyses can be assumed to present mainly the effect of RapH overproduction, as was confirmed by *lacZ* reporter assays.

The results from our DNA array analysis differ substantially from a recent report using a similar set-up, for unknown reasons (Auchtung *et al*., 2006). However, they

are in good agreement with an analysis of the transcriptional effects of a *rghR* mutation that leads to overproduction of RapG and RapH (Hayashi *et al*., 2006). Consistent with this, we found that a deletion of *rghR* not only affects competence, but also results in a sporulation-deficient phenotype (data not shown). The mechanism that regulates RghR has not been identified, but potentially, inactivation of the repressor could block both competence and sporulation through RapH. In addition to RapH, other factors play a role in ComK-dependent inhibition of developmental pathways, as a *rapH* mutation is not sufficient to bypass the abrogation of *srfA* and *spoIIA* expression upon artificial induction of *comK* (W.K. Smits, unpubl. obs.).

The transcription of *rapH* in a wild-type strain is activated by ComK. Several lines of evidence suggest that ComK is required as an antirepressor to reverse RghR-mediated repression of transcription. First, ComK-independent transcription of *rapH* can occur in an *rghR* mutant, but does not occur in wild-type cells (Fig. 2). Second, the presence of *rapH* under its native promoter on a multicopy plasmid resulting in ~15 copies per cell (pHT315-RapH2) in otherwise wild-type cells did not result in a detectable competence or sporulation phenotype, whereas this was clearly detected when a plasmid was used that was present at 50 copies per chromosome (pBS19-RapH2) (C. Bongiorni and M. Perego, unpublished). This indicates that titration of a repressor (i.e. RghR) is required to allow expression of *rapH*. When transcription was driven by the constitutive *spac* promoter in plasmid pHT315S (Worner *et al*., 2006), which is independent of RghR, results were comparable to those obtained from the pBS19-derived vectors (data not shown). While ComK is generally an activator of transcription (Hamoen *et al*., 1998; Susanna *et al*., 2004), the induction of *rapH* most likely represents the third case where the protein acts as an antirepressor, next to *recA* (Hamoen *et al*., 2001) and *comK* itself (Smits *et al*., 2007). Strikingly, in all these cases, ComK seems to be able to bind simultaneously with the repressor protein. This feature may relate to the fact that ComK binds through the minor groove of the DNA, placing the protein on a different face of the DNA helix than most other DNA-binding proteins.

In this study, we showed that RapH overproduction results in a drastic inhibition of sporulation and competence gene expression (Figs 4, 5 and 6A). A *rapH* mutant strain showed a pronounced effect on ComA-mediated *srfA* expression in a *spo0A/abrB* background (Fig. 6B). Moreover, a small but reproducible reduction in *abrB* transcription, consistent with higher levels of Spo0A~P, was observed in an otherwise wild-type strain (C. Bongiorni and M. Perego, unpubl. data). These data show that deletion of *rapH* induces the pathways leading to both competence and sporulation, and suggest that the effects of the mutation may be masked by additional positive and negative regulators. However, a clear phenotype was observed in the fluorescent microscopic analysis of a *comGA–cfp spoIIA–yfp* strain. The negative correlation between the two expression patterns observed in the wild-type strain was significantly reduced in the *rapH* mutant strain (Fig. 9) indicating an important role for RapH in maintaining the temporal separation between competence and sporulation gene expression. Previously, RapA and RapE were postulated to be of importance for the early decision to become competent or sporulate. As RapH is induced by the competence transcription factor ComK, it functions at a later stage to delay sporulation while cells are competent. Upon the escape, which might be influenced by the negative feedback imposed by RapH, cells can proceed to form an endospore. It remains to be established whether this progression occurs directly or via vegetative growth.

Both competence and sporulation require the synthesis of a complex macromolecular apparatus which is energy intensive and requires abundant resources. When cells enter the stationary growth phase neither of these is in excess, and a negative feedback loop mechanism such as the one described here serves to limit the cell to a single differentiation pathway and ensures that sporulation is indeed the last resort adaptive response.

Experimental procedures

Strains and plasmids

All plasmids were maintained in *E. coli* MC1061 or DH5α unless indicated otherwise (Sambrook *et al*., 1989). *B. subtilis* strains used in this study (Table S1) are isogenic derivatives of strain 168 or strain JH642. Plasmids and oligonucleotides used in this study are shown in Tables S2 and S3.

Mutants of *rapH* and *rghR* were constructed by replacing the ORFs with a tetracycline resistance cassette. For *rapH*, a fragment containing the upstream and downstream regions was generated using primers rapHup-F/rapHup-R and rapHdown-F/rapHdown-R respectively. Note that this fragment corresponds to the *rapH* ORF according to the sequence in SubtiList R16.1 (Moszer *et al*., 2002), but to *rapH/phrH* according to our sequencing data and Hayashi *et al*. (2006). A tetracycline resistance marker was obtained by PCR using plasmid pDG1514 as a template (Guerout-Fleury *et al*., 1995). The PCR product of the upstream region, downstream region and antibiotic marker was digested with BamHI, KpnI and both enzymes, respectively, and joined together in a three-point ligation for 1 h at room temperature. Subsequently, a PCR was performed on a 1:100 dilution of the purified ligation mixture, using PWO DNA polymerase (Roche). The PCR product was transformed directly into *B. subtilis* 168, and selected on appropriate antibiotics. Correct integration was verified by PCR analysis. A tetracycline-resistant mutant of the transcriptional regulator encoding gene *yvaN (rghR)* was obtained as follows: the

chromosomal region of *yvaN*, including the up- and downstream genes, was obtained by PCR amplification using primers yvaNup-F and yvaNdown-R. The obtained 1.8 kb fragment was cloned into the pGEM-T Easy vector (Promega), yielding plasmid pGT-yvaN, which was maintained in *E. coli* XL1BLUE. A PCR was performed on a 1:100 dilution of this plasmid using primers yvaNup-R and yvaNdown-F. The obtained product was digested with BamHI and HindIII, and ligated to a similarly digested tetracycline marker, obtained as described above, yielding pGT-yvaN-1514. Subsequently, the plasmid was transformed into *B. subtilis*. Double-cross-over integration of the construct was verified by PCR analysis. It should be noted that strain BFA1118 generated by the Bacillus Functional Analysis project (Kobayashi *et al*., 2003), listed as a *yvaN* mutant in both the Micado (http://locus.jouy.inra.fr/cgi-bin/genmic/madbase_home.pl) and BSORF (http://bacillus.genome.jp) databases, does not contain a disruption of the gene. The construct contains a fragment upstream of the *yvaN* coding sequence, and therefore it introduces a *yvaN–lacZ* reporter upstream of a native copy of the gene. Accordingly, no phenotype with respect to *rapH* or *comG* expression was observed when this strain was analysed (data not shown).

Fluorescent reporter fusions were constructed by cloning appropriate DNA fragments into plasmids pICFP, pIYFP (Veening *et al*., 2004) or pSG1151 (Lewis and Marston, 1999), pSG1186 and pSG1187 (Feucht and Lewis, 2001). A *rapH–icfp* fusion was constructed as follows: a 1615 bp DNA fragment carrying *rapH* and a 302 bp upstream sequence was generated by PCR amplification using primers rapHFP-F and rapHFP-R on chromosomal DNA of *B. subtilis* 168. The fragment was digested with EcoRI and KpnI and ligated into similarly digested pICFP (Veening *et al*., 2004). The resultant plasmid, pICFP-rapH, was checked by restriction mapping and introduced into *B. subtilis* via Campbell-type integration at the native *rapH* locus. This placed the fusion under the native promoter, and left an intact copy of *rapH* driven from its own promoter. Strain comGA–YFP(Cm) and comGA–CFP(Cm) were constructed as follows: a 594 bp fragment of the promoter region of *comG* was amplified by PCR using primers comGprom1 and comGprom2 (Smits *et al*., 2005a). After restriction with HindIII and EcoRI, this fragment was ligated into similarly digested pSG1186 or pSG1187 (Feucht and Lewis, 2001) respectively. The plasmids pSG-GACFP and pSG-GAYFP were integrated by Campbell-type isotopic integration into the chromosome of *B. subtilis*. To obtain comGA–YFP(Em) and comGA–CFP(Em), the chloramphenicol-resistant strains were transformed with pCm::Em, in a similar fashion as described for strain XH(Em). To construct plasmid pGFP-srfA, carrying the *B. subtilis srfAA* promoter region followed by a perfect ribosomal binding site fused with the *gfpmut1* gene, a PCR with the primers srfA-F and srfA-R was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with HindIII and EcoRI, and ligated into the corresponding sites of pSG1151 (Lewis and Marston, 1999). *B. subtilis* strain *srfA–gfp* was obtained by a Campbell-type integration of plasmid pGFP-srfA into the chromosomal *srfAA* promoter region of *B. subtilis* 168. Transformants were selected on TY agar plates containing chloramphenicol (5 μg ml^{-1}), after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown). Strain IIA-lYFP(Sp) was constructed by transforming a previously constructed *spoIIA–iyfp* construct (Veening *et al*., 2005) with plasmid pCm::Sp (Steinmetz and Richter, 1994) as described above.

Overexpressing of RapH, or RapH and PhrH achieved on the basis of multicopy plasmids pHT315 (Arantes and Lereclus, 1991), pHT315S (Worner *et al*., 2006), pBS19 (Band and Henner, 1984) or xylose induction (Kim *et al*., 1996). Strains XH carrying the *rapH* and *phrH* gene under a xylose-inducible promoter was constructed as follows: a 1327 bp DNA fragment was amplified using primers pXrapH-F and pXrapH-R, using chromosomal DNA of *B. subtilis* 168 as a template. The fragment was digested with XbaI/BamHI, and subsequently ligated into SpeI/BamHI-digested pX (Kim *et al*., 1996). The plasmid, pXH, was checked by restriction digestion and subsequently introduced by a double-cross-over event into the ectopic *amyE* locus of the *B. subtilis* 168 chromosome, yielding strain XH(Cm). Transformants were selected on chloramphenicol agar plates and correct integration was verified by checking the absence of amylase activity on plates containing 1% of starch. To obtain strain XH(Em), strain XH(Cm) was transformed with plasmid pCm::Em (Steinmetz and Richter, 1994), replacing the chloramphenicol marker with an erythromycin cassette. The resultant strain was checked for resistance against erythromycin and sensitivity towards chloramphenicol. Overexpression of RapH alone or RapH and PhrH from their own promoter in the multicopy vectors pHT315 (Arantes and Lereclus, 1991) or pBS19 (Band and Henner, 1984) was obtained by cloning PCR amplified fragments generated with the oligonucleotide pairs RapH5′Kpn-RapH3′Bam2 and RapH5′Kpn-RapH3′Bam3, respectively, and digested with KpnI and BamHI. Overexpression of RapH or RapH and PhrH from the *spac* promoter in plasmid pHT315S (Worner *et al*., 2006) was obtained by cloning PCR-amplified fragments generated with the oligonucleotide pairs RapH5′Kpn2-RapH3′Bam2 and RapH5′Kpn2-RapH3′Bam3. The *spac* promoter in plasmid pHT315S is constitutively expressed due to the lack of the *lacI* gene.

Escherichia coli lacZ fusion constructs were based on plasmid pJM115, a derivative of pDH32 carrying a kanamycin resistance marker in place of the chloramphenicol acetyl transferase gene (Cosmina *et al*., 1993). An *E. coli rapH–lacZ* fusion was obtained by cloning a 270 bp fragment, generated by PCR amplification using oligonucleotide primers RapH5′Kpn-RapHprom3′Bam, and digested with BamHI in the SmaI–BamHI sites of pJM115. This fragment contains 215 bp upstream of the start codon of the *rapH* gene. The transcriptional fusion of the *comG* promoter to the *E. coli lacZ* gene was constructed using a fragment generated by PCR amplification, with oligonucleotide primers comG5′ and comG3′. The 329 bp fragment digested with EcoRI and BamHI was cloned in the similarly digested pJM115 vector and the resultant plasmid used to transform strain JH642 generating strain JH11205 upon double-cross-over integration in the chromosomal *amyE* locus. The *srfA–lacZ* transcriptional fusion was constructed by cloning a 720 bp fragment obtained by PCR amplification using oligonucleotide primers srfApromEco and srfApromBam. The promoter-carrying fragment was cloned in the EcoRI–BamHI restriction sites of

vector pJM115. The plasmid was transformed into *B. subtilis* JH642 generating strain JH11694 upon double-cross-over integration in the chromosomal *amyE* locus.

Strains containing mutations at multiple loci were obtained by transformation with chromosomal DNA from the single mutant strains. In the case of the reporter strains, DNA containing mutations was introduced in the reporter strain, assuring the same genetic background for the flow cytometric analyses and fluorescent microscopy.

Fidelity of PCR amplification was checked by DNA sequencing analysis; two discrepancies (Hayashi *et al.*, 2006) within the *rapH-phrH* region with the sequence deposited in SubtiList were confirmed also in the genome of strain JH642.

Media and growth conditions

Both *B. subtilis* and *E. coli* strains were routinely grown in TY or LB, supplemented with selective antibiotics when appropriate. TY consist of 10 g l^{-1} tryptone (Difco), 5 g l^{-1} yeast extract (Difco) and 5 g l^{-1} NaCl, pH 7.4. In addition, *B. subtilis* was grown in Spizizen minimal medium (Anagnostopoulos and Spizizen, 1961) to induce competence, and on sporulation medium (SM) (Schaeffer *et al.*, 1965) to induce expression of sporulation genes. To induce *rapH* expression from the Pxyl promoter, cells were grown in fructose minimal medium (MMF) (Smits *et al.*, 2005b), and SM medium supplemented with 1% xylose. Finally, to allow expression of competence and sporulation genes in the same culture, *B. subtilis* was grown in a chemically defined medium supporting both processes (dubbed CDMLG), which was adapted from a previously described medium (Veening *et al.*, 2006). The medium contains 5 mM $MgSO_4 \cdot 7H_2O$, 40 mM 3-(N-morpholino-) propanesulphonic acid (MOPS; pH 7.1), 4 mM KH_2PO_4, 9.5 mM $(NH_4)_2SO_4$, 6 mM sodium-L-glutamate, 0.15% glucose, 1× MT mix (Vasantha and Freese, 1980) and auxotrophic requirements (0.1 mM L-tryptophan). Note that in this medium, competence development occurs from late exponential growth phase to stationary growth phase, even when cells were pre-cultured in a rich medium. Antibiotics were used at the following concentrations: chloramphenicol 5 μg ml^{-1}; erythromycin and lincomycin 5 μg ml^{-1} and 25 μg ml^{-1}, respectively; kanamycin 2 μg ml^{-1}; spectinomycin 50 μg ml^{-1}.

Protein expression and purification

Expression of RapH in *E. coli* was obtained from plasmid pET28 (Novagen) by cloning the *rapH* coding sequence, generated by PCR amplification using oligonucleotide primers RapH5′Bam-RapH3′Bam2, in the BamHI site of the vector thus generating a fusion to six histidine codons at the 5′ end of the gene. The resulting plasmid, pET28-RapH, was transformed in the *E. coli* expression strain BL21(DE3)pLysS (Novagen) and cells were grown at 37°C in M9ZB medium (Studier *et al.*, 1990) until the density reached $OD_{600} = 0.5$. The culture was transferred to a 16°C shaker incubator and protein expression was induced by the addition of 0.2 mM IPTG. Cells were grown for 18 h before harvesting by centrifugation. Cell pellet was re-suspended in 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM imidazole and 1/1000 dilution of Protease Inhibitor Cocktail (Sigma). Cells were lysed with a French press and the lysate was ultracentrifugated at 45 000 r.p.m. in a Type 60TI rotor for 1 h. The clear lysate was recovered and incubated for 1 h with Ni-NTA agarose (Qiagen) before loading it into a column. The column was then washed with the cell re-suspension buffer before eluting the RapH protein with a step gradient of imidazole. Fractions containing the protein were pooled and dialysed against 50 mM HEPES pH 7.2, 200 mM NaCl, 10 mM DTT. Glycerol was added to 30% final concentration and the protein was stored at −20°C.

Expression of DegU in *E. coli* was obtained from plasmid pET20b (Novagen) by cloning the *degU* coding sequence generated by PCR amplification using oligonucleotide primers DegU5′ and DegU3′ in the NdeI–XhoI sites of the vector. This resulted in the fusion of six histidine codons to the 3′ end of the *degU* gene. Plasmid pET20-DegU was transformed in the *E. coli* expression strain BL21(DE3)pLysS (Novagen) and cells were grown at 37°C in LB supplemented with ampicillin. At $OD_{600} = 0.7$ protein expression was induced by the addition of 3 mM IPTG and cells were grown for an additional 2 h at 37°C. Harvested cells were re-suspended in 50 mM potassium phosphate buffer pH 7.8, 30 mM NaCl, 5 mM β-mercaptoethanol, 1 mM PMSF. Cells were lysed by sonication and the lysate was centrifuged for 30 min at 27 000 *g*. The clear lysate was recovered and incubated with Ni-NTA agarose (Qiagen) for 2 h at 4°C before pouring into a column. The column was washed with the cell re-suspension buffer before eluting the DegU protein with a step gradient of imidazole. The protein was stored at −20°C in the elution buffer supplemented with 25% of glycerol.

His6-YvaN was isolated from an *E. coli* strain harbouring the pHis-YvaN plasmid (Hayashi *et al.*, 2006) as follows. An overnight culture was diluted 1:100 into fresh TY with appropriate antibiotics. Growth was continued until OD 0.70 under continuous shaking (250 r.p.m., 37°C). At that moment, expression of His6-YvaN was induced by the addition of 1 mM IPTG, and continued for 1 h. Subsequently, cells were pelleted by centrifugation (10 min, 8000 r.p.m., 4°C), and stored at −80°C. The pellet was re-suspended in 5 ml of buffer A (20 mM Tris-HCl pH 8.0, 0.2 M NaCl, 10 mM $MgCl_2$, 7% glycerol, 1 mM β-mercaptoethanol, 5 mM imidazole), supplemented with Complete Mini Protease Inhibitor (Roche), and cells were disrupted by sonication. Cellular debris was removed by centrifugation (10 min, 14000 r.p.m., 4°C), and the supernatant fraction was incubated with 2 ml of equilibrated Superflow NiNTA resin (Qiagen) in a total volume of 15 ml of buffer A for 2 h under continuous mixing. The column material was packed in a Poly Prep Chromatography Column (Bio-Rad) and washed by gravity flow with 30 column volumes buffer A and 30 column volumes buffer B (identical to buffer A, but with 20 mM of imidazole). The protein was eluted from the column with buffer C (identical to buffer A, but with 500 mM imidazole), and 0.5 ml fractions were collected. Fractions were checked for protein content and purity by SDS-PAGE. Soluble protein was quantified using the RC/DC protein determination kit (Bio-Rad), using a commercial bovine serum albumin solution (New England Biolabs) as a standard.

Purification of Spo0F, Spo0F~P, ComA, ComA-N-terminal domain, ComA-C-terminal domain, RapA and ComK were carried out as previously described (Hamoen *et al*., 1998; Ishikawa *et al*., 2002; Bongiorni *et al*., 2005; Susanna *et al*., 2006).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) with ComK were performed essentially as described (Albano *et al*., 2005). In short, a 343 bp DNA fragment comprising 302 bp of the upstream region of *rapH* was amplified using primers PrapHFP-F and RapHFP-R and Extensor polymerase (ABgene). The resulting fragment was end-labelled with T4 polynucleotide kinase (Roche) and [γ-^{32}P]-ATP, and pre-mixed with ComK protein on ice. For comparison a region upstream of the *comGA* ORF (Albano *et al*., 2005) was included. After incubation for 15 min at 37°C free probe and complexes were separated on a non-denaturing 6% polyacrylamide gel, in a 0.5–2× TAE gradient, dried and autoradiographed. The EMSA with ComA was carried out as described in Core and Perego (2003).

Alignment and construction of evolutionary tree

Amino acid sequences of RapA, RapB, RapC, RapD, RapE, RapF, RapG, RapI and RapJ were derived from SubtiList (http://genolist.pasteur.fr/SubtiList/) (Moszer *et al*., 2002). The RapH amino acid sequence was based on published information (Hayashi *et al*., 2006) and DNA sequencing results. Proteins were aligned using CLUSTALW (Thompson *et al*., 1994) and an evolutionary tree was generated using Treecon software (Van de Peer and De Wachter, 1994) as described before (Van Hijum *et al*., 2002). Treecon output was prepared for publication using Corel Graphics Suite 11.

Fluorescence microscopy

Fluorescence microscopy of fluorescent reporter strains was carried out essentially as described (Smits *et al*., 2005a; Veening *et al*., 2004). To quantify fluorescent signals from individual cells, captured images were imported into Quantity One software (Bio-Rad). A grid for all cells was generated based on the phase-contrast image, and subsequently overlaid on unprocessed images from the CFP and YFP channels. Data from both channels were combined in Microsoft Excel for further analysis and graphs for publication were prepared in Corel Graphics Suite 11.

Reporter analyses

Single-cell analyses of the GFP reporter strains were performed as described before, based on a flow cytometric analysis of 20 000 cells per sample (Smits *et al*., 2005a). Cultures for β-galactosidase assays were grown at 37°C in Schaeffer's sporulation medium (Schaeffer *et al*., 1965) or in Spizizen minimal medium (Anagnostopoulos and Spizizen, 1961) with the appropriate antibiotics. Samples were taken at hourly intervals; cells were harvested by centrifugation, re-suspended in equal volume of Z buffer (Miller, 1972) and incubated at 37°C for 5 min with 100 μg ml^{-1} lysozyme. After treatment at room temperature with 0.1% Triton X-100, samples were assayed for β-galactosidase activity by the method of Miller (1972). The results shown are representative of at least two independent experiments.

Phosphatase and native gel protein binding assay

The phosphatase activity of 6xHis-RapH against Spo0F~P was assayed essentially as described before (Bongiorni *et al*., 2006).

The native polyacrylamide gel electrophoresis binding assay was carried out essentially as previously described (Bongiorni *et al*., 2005). Gels were run for 36 h at 60 V (constant voltage) at 4°C.

DNA array analyses

To assess global transcriptional profiles, we employed in-house developed microarrays, representing all ORFs of *B. subtilis* and several additional control genes. Details about the design of these arrays is described elsewhere (Lulko *et al*., 2007). Data were analysed essentially as described before (Den Hengst *et al*., 2005). In short, three independent cultures per medium type were grown for parental and *rapH*-overproducing strains. Each ORF is represented duplicate spots on the array. The indirect labelling of 20 μM of total RNA included a dye-swap to compensate for possible dye-specific effects. After hybridization, fluorescent signals were quantified with ArrayPro analyser, and processed with MicroPrep (Van Hijum *et al*., 2003). Statistical analysis was performed using CyberT (Baldi and Long, 2001; Long *et al*., 2001). Genes with a Bayes *P*-value below 1.0×10^{-4} with at least twofold differential expression were considered to be significantly affected. Raw and normalized data as well as a hybridization scheme of these experiments can be obtained from http://molgen.biol.rug.nl/publication/rapH_data/.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. ComK and RghR can bind simultaneously to the promoter of *rapH*.

A. Sequence of the upstream region of *rapH* and the start codon (white box, start) are derived from SubtiList (http://genolist.pasteur.fr/SubtiList/). Putative binding sites for RghR (RghR, in yellow), as well as the core promoter elements (−35 and −10, in grey) and transcriptional start site (+1), were determined by Hayashi *et al*. (2006). The putative ComK binding site (K), composed of two AT boxes (AT1 and AT2), is postulated on the basis of the published consensus sequence (Hamoen *et al*., 1998) and the position compared with the core promoter elements (Hamoen *et al*., 2002). It has to be noted that another ComK binding site can be identified that overlaps the promoter elements (Berka *et al*., 2002; Hamoen *et al*., 2002).

B. Electrophoretic mobility shift assays of a [γ-^{32}P]-ATP-labelled *rapH* promoter fragment in the presence of purified ComK and/or RghR. Grey bars indicate shifted complexes of DNA and protein, small triangles indicate supershifted complexes. A black bar indicates free probe. X marks the lane to which no protein was added. ComK was added to a final concentration of 300 nM.

Fig. S2. Analysis of the genome-wide transcriptional effect of RapH/PhrH overexpression in strains isogenic with 168 (*trpC2*) (Kunst *et al*., 1997). Genes significantly affected in a CyberT analysis (see *Experimental procedures*) were analysed using FIVA software (Blom *et al*., 2007).

A. Effect of RapH/PhrH overproduction in Spizizen minimal medium.
B. Effect of RapH/PhrH overproduction in Schaeffer's sporulation medium.

Fig. S3. Time-course analysis of *abrB–gfp* reporter strains grown in MMF medium supplemented with 1% xylose as indicated in *Experimental procedures*.
A. Wild-type strain.
B. Strain XH, ectopically overexpressing RapH/PhrH from a xylose-inducible promoter. Strains were grown in the presence (grey) or absence (red) of xylose. Colours are darker at later time points. Note the downregulation of *abrB* transcription, as indicated by lower levels of fluorescence, in wild-type or uninduced XH strains.

Fig. S4. RapH interacts with ComA and inhibits its DNA-binding activity.
A. RapA does not interact with ComA in the native gel binding assay. Each protein was at 12 μM final concentration.
B. RapA does not inhibit the DNA-binding activity of ComA. X: labelled probe only; RapA (lane 2: 5 μM, triangle: 5, 10 and 20 μM); ComA (5 μM).
C. RapH does not interact with DegU in the native gel binding assay. H: RapH; U: DegU; each protein was at 10 μM final concentration. Native gel analysis was carried out on 10% native Tris-Tricine gels as described in Bongiorni *et al.* (2005).

Fig. S5. Time-course of β-galactosidase activity of a *srfA–lacZ* (A) and a *rapA–lacZ* (B) reporter constructs in the following background strains: wild type (-■-); *spo0A* (-●-); *spo0AabrB* (-◆-). Strains were all isogenic to JH642 (*trpC*2, *phe*-1). Cells were grown in Schaeffer's sporulation medium. Samples were taken at hourly or half hourly intervals to represent the time of transition from exponential growth to stationary phase.

Table S1. *B. subtilis* strains used in this study.

Table S2. Plasmids used in this study.

Table S3. Oligonucleotide primers used in this study.

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